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(54) Title: A METHOD FOR THE PURIFICATION OF IMMUNOGLOBULINS

(57) Abstract

The present invention concerns a method for the purification of immunoglobulins from a source solution by use of a cation-exchange resin.

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A METHOD FOR THE PURIFICATION OF IMMUNOGLOBULINS

FIELD OF THE INVENTION

The present invention is generally in the field of purification of immunoglobulins from a source solution and more specifically concerns purification of immunoglobulins utilizing ion-exchange resins.

BACKGROUND OF THE INVENTION

Therapeutic and prophylactic specific and non specific immune serum globulins (ISG) preparations are well known and have been available for many years. These preparations are used in passive immunization and in the treatment of a variety of infections. ISG is presently obtained in commercial quantities using variations of a blood plasma fractionation technique developed by Cohn *et al.* in the 1940's based on a cold ethanol precipitation technique (McCue *et al., Review of Infectious Diseases*, 8(4);5374-5381, (1986)). ISG has been administered intramuscularly (IM) and more recently intravenously (IV), the latter method of administration provides numerous advantages and has gained acceptance as the preferred method of administration. Initial attempts to render ISG safe and effective for IV administration (IVIG) focused on eliminating its anticomplement activity. The production of clinically safe and efficacious intravenous Immunoglobulins (IVIg) without side reactions which derived either from the formation of aggregates, the presence of anti-complement activity or form the prekalikrein activator was behind the driving force to produce improves formulation based on modification of the production procedure.

The final formulation has been supplemented in various commercial product with 10% sucrose, glucose, PEG or Tween 80. However, all the products containing salt were lyophilized and had to be reconstituted before use. Only a few products are in a liquid form. These products have very low ionic strength and are supplemented with 10% maltose. The final pH ranges from 4.2-5.5. During the 15 year of production of IVIg, the trend of manufacturing has been to move out of either chemically modified molecule by acid treatment (Uemura, Y., *J. Exp. Med.*, 141(3):337-349 (1983)) or by enzymatic digestion by trypsin in low pH. All these modifications were proved to be inferior to natural intact Immunoglobulins.

Although ISG products (both IMIG and IVIG) have been considered generally safe, there has been a growing need to assure patients that ISG products do not transmit active viruses such as those associated with hepatitis or, HIV which is associated with Acquired Immune Deficiency Syndrome (AIDS). There are many citations in the literature that Cohn fraction II, the main source of Immunoglobulins used in the 80s was contaminated with Hepatitis C. Based on the above findings, a new method for virus inactivation was developed by the New York Blood Center. The method is based on the virucidal effect of an organic solvent combined with an nonionic detergent (US 4,481,189 and US 4,315,919). The high amount of both the detergent and the organic solvent is toxic, therefore both have to be removed from the final formulation.

Various ways were designed to remove the impurities of the solvent and detergent from the formulation, for example, EP A-0 366346, EP -A0 239859, EP-A-0 322786 and EP-A- 0 131 740 (Horowitz *et al.*) disclose using mineral oil for purification purposes. In another publication the solvent was removed by mineral oil and the detergent Triton X-100 was removed by C-18 hydrophilic column (EU 92111947 to Gehringer *et al.*).

Another approach for removal of the organic solvent and the nonionic detergent was by absorbing the immunoglobulins on an anion exchange resin such as DEAE (McCue *et al., Supra*; Hans R. Friedli, *Pharmacotherapy Supp.*, 7(2):36-40, (1987)). During the absorption process the pH of the resin has to be adjusted to a pH of about 7-9. The purification by anion exchange resins has the disadvantages of loss of IgG sub-classes 3 and 4, as compared to the source solution. In addition purification by anion exchange resins causes the purified gamma-globulins to aggregate, which aggregates have to be eventually disassociated by digestion with trypsin and pepsin, resulting in an Ig molecule of reduced efficacy. The yield of purification by anion exchange resins is usually in the range of 75 to 85% since a large amount of the immunoglobulins are not absorbed on the anion exchange resin and are thus discarded.

A slight modification of the purification by anion exchange resin utilizing a single column DEAE Sephendex (procedure was developed A.D. Friesa *et al., Vox Sang*, 48:201-212 (1985)) wherein the purification proceeds by sequential chromotographies on two cross-linked agarose gel anion exchanges, thus enabling repeated *in column* recycling. However, the other disadvantages of purification of immunoglobulins by anion exchange resins, namely, loss of IgG subclasses 3 and 4 and aggregation are evident also in this modification.

It would have been highly desirable to provide a method for purifying immunoglobulins which would give a high yield of immuno-globulins, produce purified immunoglobulins having an IgG sub class 3 and 4 ratio which is similar to the ratio in the source solution and which method would not cause aggregate of the purified immunoglobulins.

SUMMARY OF THE INVENTION

The present invention is based on the surprising finding that cation exchange resins, preferably pre-conditioned with a pH lower than 4.5, can leave a retinue of relatively large quantities of immunoglobulins as compared to anionic

resins. According to the finding of the invention, it was discovered that by using cation-exchange resins, essentially all the solvent/detergent was eliminated. A further finding was that the elution at higher salt concentration and pH above 7.5 releases (eluted) all the immunoglobulins G heavy chain subclasses, notably IgG subclass 3 and 4, so that the purified immunoglobulins have a sub-class ratio similar to that of the source solution.

Thus, the present invention provides a method for the purification of immunoglobulins from a source solution comprising:

- contacting the source solution with a cation exchange resin; and
- eluting the immunoglobulins bound to said cation exchange resin.

The term *"immunoglobulin"* refers to immunoglobulins in particularly IgG of all sub-classes.

The purification may be from any source solution such as: Cohn Fraction Paste II, PEG precipitated plasma supernatant, DEAE purified plasma effluent, ammonium sulfate precipitate of plasma or serum.

Preferably, the purification is from a Cohn Fraction III, i.e. from a fraction obtained from blood plasma by a cold ethanol precipitation technique as specified in Technical Procedure I, hereinafter.

The method comprises contacting the source solution with a cation exchange resin. The term *"cation exchange resin"* refers to a highly polymerized synthetic organic compound consisting of a large, non-diffusable anion and a simple, diffusable cation, which latter can be exchanged for a cation in the medium in which the resin is placed. Examples of cation exchange resins are any kind of matrix functionalized with a carboxy, sulfo, sulfoalkyl, cyano and phenyl groups. The cation exchange compound may be bound to any type of solid support including any matrix resins.

Preferably, the cation exchange resin is constructed in the form of a chromatographic column and the method includes passing the source solution

through the cation exchange chromatographic column which leads to binding of the immunoglobulins to the cation exchange resins.

Preferably, prior to contact of the source solution with the cation exchange resin, for example prior to passing the source solution through the cation exchange chromatographic column, the resin is pre-treated with an acidic solution. In accordance with the present invention, it was surprisingly found that such pre-treatment facilitates binding of the immunoglobulins to the resins, increases the capacity of the cation exchange resin, and the yield of the whole process, from about 30 mg per ml of resin to 50 mg of SP resin.

The concept of utilizing pof the ion-exchange resin by acidic solution, is contrary to the state of the art concept of using neutral ion exchange membranes. Ion exchange resins are usually cleaned by acid followed by base or *vice versa*. However, in most of the procedures for protein purification the resin is equilibrated before loading the protein with neutral or very close to neutral pH. This has been done in order to avoid any harm to the protein. Inventors of the present invention have surprisingly found that this equilibration is in very narrow margins. pHs lower than 3.5 caused the immunoglubulin to be absorbed on the resin without ability to release it even with high salt at high pHs. However, at a pH between 3.8 to 4.5 elution was close to 100% of the loaded protein without forming aggregates. Equilibration at subsequently higher pH causes reduction of the amounts of immunoglobulins absorbed to the column. The pre-treatment should be by rinsing the cation exchange resin with a solution having a pH of 3.5-5.5, preferably 4.0 to 4.5, most preferably of 4.5.

The immunoglobulins are removed or *"eluted"* from the cation exchange resin, by rinsing it with a solution having a high salt concentration, usually in the range of 0.3 molar 1.5 molar, preferably 0.45 to 0.65 molar, most preferably 0.5 molar and having a pH in the range of 6.0-9.0, preferably 6.5 to 7.5, most preferably a pH of 7.0.

In accordance with the present invention, it is preferable to treat the source solution, prior to contact with a cation exchange resin, with an nonionic detergent and an organic solvent, in order to inactivate viruses present in the source solution, as specified in U.S. 4,481,189, and U.S. 4,315,919, the contents of which are incorporated herein by references. Examples of organic solvents are: Tri-n-Butylphosphate or Tri-n-Propylphosphate. Examples of nonionic detergents are: Triton X-100, Tween 20, Tween 80, sodium cholate and NP-40. Preferably, the source solution should be incubated with a solvent/detergent in an acidic pH, at a pH in the range of 5.1 to 5.4, preferably a pH of 5.2 for several hours at low temperatures such as 4°C.

The cationic exchange chromatography serves to remove virtually all the solvent/detergent reagents. However, if it is desired to remove also various hydrophobic lipids, or lipoprotein impurities which are present in the source solution, it is possible also to pass the immunoglobulins eluted from the cation exchange resins through a hydrophobic column, preferably a hydrophobic column having a substantially smaller volume than the volume of the cation exchange column, for example, 7 to 12 times smaller preferably about 10 times smaller.

More specifically, the present invention provides a method for the purification of immunoglobulins from Cohn Fraction III solution comprising:

(i) diafiltratring the Cohn Fraction III solution against water;

(ii) adjusting the pH of the diafiltrated solution to a pH of 5.1 to 5.4;

(iii) contacting the solution obtained in (ii) with an organic solvent and a detergent;

(iv) passing the solution obtained in (iii) through a cation exchange chromatographic column;

(v) eluting the immunoglobulins absorbed on the column by passing a solution having a salt concentration of 0.3 M - 1.5 M and a pH of 6.0-7.0.

(vi) diafiltrating the elute of step (v) against water; and optionally

(vii) passing the eluted immunoglobulin obtained in step (vi) through a hydrophobic column which is 7 to 12 times smaller than the cation exchange column.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows a schematic flow chart for the preparation of Cohn Fraction III.

DETAILED DESCRIPTION OF THE INVENTION

TECHNICAL PROCEDURE I

The following is a general flow chart for the preparation of an immunoglobulin containing fraction beginning from frozen plasma:

Omr-IgG-am IV Production Flow Chart

Production steps	Conditions	# Sample
Frozen plasma pooled plasma	Thawing	A1
cryoprecipitate	Centrifug.: 3-5 1/min.	A2
Cryosupernatant	Temperature 3 ± 1°C Protein Adjustment	A3
Separation TI precipitation	Ethanol: 8%, pH 7.2 Temperature –2 ± 0.5°C	A4
centrifugation	Fibrinogen production	A5
Separation TII+III precipitation	Ethanol 20%, pH 6.85 Temp. –5 ± 1°C. Stir. 3h.	
		A6
alluvial filtration	Harbolite Supercell	
Supernatant, I, I, III (Following Albumin process)	postwash 1: temp. -5°C pH 6.85, Ethanol 20% NaCl 110 mM;	
	postwash 2: pII 7.5, Ethanol 20% Sod. Phosphate dibasic 2.2 mmol	
PASTE I, II, IIIw (frozen at -25°C) **Fraction I, II, IIIw**	water resuspension Temp. 1 ± 1°C. pH 5.2 Stirring at least 3 h. Sod. Acetate 0.04M Stirring 2 h.	
		B1
	Ethanol 17% Temperature -5°C	B2
	pH 7.4 (Sod. Barcarb.)	B3

Fig. 1 shows a schematic representation of the preparation of Cohn Fraction III.

EXAMPLES

Example 1 The effect of various buffers used for conditioning and elution on the concentration of IgG purified on an SP column

A. Technical procedure

10 L of Cohn fraction III Batch No. GoFooAN / B262551 produced by Omrix biopharmaceutical Plasma Fractionation Institute (Rehovot, Israel) was concentrated to 550 ml by ultrafiltration and then dialyzed against 6 volumes of DDW to reduce the salt and the ethanol concentration. Lipid coated viruses were inactivated by addition of 0.3% Tri-n-butyl-phosphate and 1% triton-x-100. The mixture was incubated at 4°C for 4 hours, after which the solution was diluted to 50 mg per ml (Table 1).

Table 1

Preparation of concentrate Fraction III from Batch No. GoFooAN/ B262551 with solvent detergent (SD) for the purification on a SP column

Sample No.	Volume	Protein con.	Total protein	Recovery
	ml	mg/ml	mg	%
Fraction III filtrate (From Cohn fractionation)	11,000	4.19	46,090	100.00
Ig04 (concentrate of Frac. III)	550	83.8	46,090	100.00
Ig04 after filtration 1.2U	548.5	79.7	43,715	94.85
Ig04 after SD	564.5	79.3	44,765	97.12
Ig06a after dilution to <50 mg/ml (diluted with WF1)	890	50.4	44,856	97.32
Ig06b after filtration 0.45 µm	880	49.5	43,560	94.51

The combined mixture was loaded on a pre-conditioned FPLC (Pharmacia, Sweden) mounted with a 16 mm column loaded with 74 ml Fractogel SP (TosoHaas, Japan) resin, which is a hydrophilic methacrylate copolymer matrix functionalized with a sulphopropyl ($-CH_2CH_2CH_2SO_3$) group. This 470x16 column as the same bed height as the large scale column. The Fractogel SP column was pre-conditioned with 2 column volumes of various buffers as specified in Table 2 below, the Immunoglobulin (concentrated Cohn Fraction III prepared according to Fig. 1) mixture with the solvent detergent reagent was loaded at a rate of 1 ml/min. followed by running various elusion buffers as specified in Table 2 below. After the elusion of the SD from the mixture the protein was eluted from the SP column by various elution buffers (see Table 2).

Table 2

Various combination of preconditioning and elusion buffer used to load and elute a mixture of Cohn fraction III concentrate +SD on a Fractogel SP column

Run Name	Column Conditioning	Column elution	Elution Volume
IGA1	Citric acid pH 4.0	0.45 M NaCl + 50 mM sodium citrate pH=7.0	124.00
IGA2	Citric acid pH 4.0	0.45 M NaCl + 50 mM sodium citrate pH=9.0	143.00
IGA3	Acetic acid pH 4.0	0.45 M NaCl + 50 mM glycine pH 9.0	226.00
		0.45 M NaCl + 50 mM glycine pH 9.0	
IGA4	Citric acid pH 4.0	0.45 M NaCl + 50 mM ammonium hydroxide pH 9.0	133.50
IGA5	Citric acid pH 4.0	0.45 M NaCl + 50 mM sodium citrate pH = 7.0	128.00
IGA6	Citric acid pH 4.0	0.45 M NaCl + 50 mM ammonium hydroxide pH 11	149.00
IGA7	Citric acid pH 4.0	0.45 M NaCl + 50 mM sodium citrate pH=7.0	126.00
IGA8	Citric acid pH 4.0		136.50

Results

The results obtained utilizing the preconditioning and elution buffers of Table 2 are summarized herein below in Table 3.

Run No.	Sample No.	Volume	Protein conc.	Total protein	recovery (%)
IGA1	Ig06b	77	49.5	3,812	
	column spend	65.5	0.011	1	
	Ig07	124	30.6	3,794	99.55

Run No.	Sample No.	Volume	Protein conc.	Total protein	recovery (%)
IGA2	Ig06b	77	49.5	3.812	
	column spend	65.	0.006	0	
	Ig07	143	26.7	3,818	100.17

Run No.	Sample No.	Volume	Protein conc.	Total protein	recovery (%)
IGA3	Ig06b	77	49.5	3,812	
	column spend	68	0.006	0	
	Ig07	226	2	452	11.86

Run No.	Sample No.	Volume	Protein conc.	Total protein	recovery (%)
IGA4	Ig06b	77	49.5	3,812	
	column spend	69.5	0.016	1	
	Ig07	133.5	29.5	3,938	103.33

Run No.	Sample No.	Volume	Protein conc.	Total protein	recovery (%)
IGA5	Ig06b	77	49.5	3,812	
	column spend	70	0.018	1	
	Ig07	128	29.6	3,789	99.40

Run No.	Sample No.	Volume	Protein conc.	Total protein	recovery (%)
IGA6	Ig06b	77	49.5	3,812	
	column spend	70.5	0.017	1	
	Ig07	149	25.7	3,829	100.47

Run No.	Sample No.	Volume	Protein conc.	Total protein	recovery (%)
IGA7	Ig06b	77	49.5	3,812	
	column spend	71	0.017	1	
	Ig07	126	30.6	3,856	100.16

Run No.	Sample No.	Volume	Protein conc.	Total protein	recovery (%)
IGA8	Ig06b	77	49.5	3,812	
	column spend	70	0.017	1	
	Ig07	136.5	29.1	3,972	104.21

(Column spend is the fluids which are not bound to the resin and which run through the column while loading the solution onto the column.)

As can be seen from the above Table, purifying immunoglobulins on a cation exchange resin SP Fractogel column resulted in a high recovery yield if appropriate pre-conditioning and elution solutions were used. It was found that acetic acid can not replace the citrate as a conditioner for the SP fractogel column, and that increasing the pH with various buffers does not significantly change the elution volume (one usually is trying to keep pH low as possible). Alkaline buffers did not improve the procedure and should be avoided in order to ensure that the IgG is not harmed, since it is well established that high salt concentrations and high pH causes aggregation of immunoglobulins.

Example 2 The effect on recovery and elution volume of Immunoglobulin G using three types of cationic exchange resins

A. <u>Technical procedure</u>

1.75 g of Cohn fraction III produced by Omrix was mixed 0.3% Tri-n-butyl-phosphate (TNBP) and 1% triton-x-100. The mixture was incubated at 4°C for 4 hours, after which the solution was diluted to

50 mg/per ml with WFI. The mixture was than loaded on a 35 ml (50 mg protein per ml of resin) column containing one of the following cation-exchange resins.

1. CM0511-CM-Toyopearl 650(M) - from Tosoh corporation Tokyo, Japan - CH_2COO (carboxymethyl).
2. SP0611- SP-Toyopearl 650 (M) - from Tosoh corporation Tokyo, Japan - $CH_2CH_2CH_2SO_3$ (sulphopropyl).
3. SPM0711- SP Sepharose Fast Flow form Pharmacia Uppsala, Sweden (sulphopropyl).

All three columns were conditioned with 50 mM citric acid pH 4.0 and the protein was eluted with 50 mM sodium Citrate and 450 mM sodium chloride.

B. Results:

The results are shown in Table 4 below.

Table 4

Resin Type	volume	recovery (%)
CM0511	55	86.08
SP0611	47	106.51
SPM0711	56	103.750

As can be seen, the recovery in the SP resin was better than the CM resin. The lower elusion volume was found in the SP-Toyopearl 650 (M) from Toso.

The ionic strength of the cationic residue has a significant effect on the gel capacity. The operating mechanism of ion exchange chromatography is by reversible bidning of charged molecules. Binding

strength is governed by the degree of charge on the substrate - the pKa of the ion exchange matrix, and the aqueous solution properties pH and ionic strength. There are four principle types of ion exchangers: strong anion, weak anion, strong cation and weak cation. This assortment of matrices recognizes the specific ionization properties (pI) that differentiate one protein from another and also distinguish localized charge differences of proteins with the same pI.

The pKa of SP resins is 2.3 and for all the CM resin is 4.2. The ion exchange capacity is 0.08-0.12 meq/ml resin for CM resin and 0.13–0.17 meq/ml resin for the SP resin.

Example 3 Recovery of Immunoglobulin G Heavy Chain Subclasses in a cationic exchange chromatography process

A. Technical procedure

Five manufacturing processes of the full immunoglobulin purification procedure starting from Fraction III filtrate and ending up in a final container containing 5% protein were tested for the Immunoglobulin G subclasses of the heavy chain by using the immuno-diffusion antigen test by ICN USA (IgG AccraAssay form ICN Biomedicals, USA). In each procedure a simple agarose immunodiffusion assay was carried out against a specific antigen against all of its classes. The same process was used in each of the runs in order to establish reproducibility.

B. Results

Recoveries of the various subclasses were close to 100% as compared to the starting material Fraction III filtrate (See Table 5).

Table 5

Recovery of IgG heavy chain subclasses in the process of IVIg

	IgG1	IgG2	IgG3	IgG4
RUN 67	107.03	78.42	127.27	100.00
RUN 16	98.78	106.27	118.18	100.00
RUN 19	93.43	111.92	109.09	100.00
RUN 20	107.03	115.29	127.27	100.00
RUN 21	95.72	92.55	113.64	80.00
Mean	100.40	100.89	119.09	96.00
SD	6.35	15.27	8.13	8.94

As can be seen, the subclasses IgG distribution of the immunoglobulins in the preparation purified on the SP resin is the same as in the starting material indicating that the purification method of the invention, utilizing cation exchange resins, does not result in loss of IgG subclasses 3 and 4. This being in contrast to a significant IgG subclass 3 and 4 loss evident in purification procedures utilizing anion-exchange resins.

Example 4 Removal of triton x-100 by chromatography on a SP-Toyopearl 650

A major factor in choosing purification techniques is the ability of the technique to completely remove essentially all of the detergent, (such as X-100), which was added in order to eliminate viruses.

A. <u>Technical procedure</u>

Intravenous immunoglobulin solution was prepared from frozen human plasma by means of Cohn method Fraction III followed by additional purification and virus inactivation steps (McCue *et al., supra*).

Fraction III which contains about 3-5 mg/ml protein and 17% ethanol was concentrated by Diafiltration to 60-70 mg/ml and the salt and the ethanol were removed by 6 volumes of Diafiltration against water. The pH was adjusted to 5.2 and solution was treated by solvent / detergent (S/D) solution comprising: 0.3% Tri (n-butyl) phosphate, 1% Triton X-100 for 6 hours at 4°C. The S/D reagents were removed by cationic exchange chromatography on a 200 ml SP Toyopearl 650 resin (Tosohaas Corporation, Tokyo, Japan ($-CH_2CH_2CH_2SO_3$) sulphopropyl functional group) at low pH (4.0). At this pH, the positively charged IVIg is bound to the column, allowing the removal of the >97 of the Triton X-100 in the column spend.

The IVIg was removed from the cationic resin by increasing the salt concentration to 0.35 molar sodium chloride and increasing the pH to 7.0. Removal of salt and concentration of protein to about 70 mg/ml was than followed by Diafiltration against water.

In order to remove the lipids or lipoproteins from the IVIg solution the solution was passed through a an hydrophobic column (C-18 resin) which is 10 times smaller than the cationic column or with a ratio of 500 mg protein per ml of resin. The IVIg protein was then incubated for 22 hours at 37°C at pH 4.0 in the presence of 10% maltose in order to reduce the aggregates of Immunoglobulins. This preparation was than subjected to an addition Diafiltration against water followed by final formulation of the balk material. The final formulation consists of 10% maltose and 50 mg/ml protein at pH 5.2 . The sterile filtered bulk product is then filled in 50 ml aliquots which was stored at room temperature.

B. Results

The experiment was conducted in two replicates. It can be noted in the results demonstrated in Table 6 below that the SP column removed more than 97% of the detergent triton x-100 from the product.

Additional 1-2% was removed by the Diafiltration. The removal by the hydrophobic column was negligible.

Table 6

Removal of triton x-100 from Immunoglobulin preparation.
(Upper table RUN20 and the lower RUN21)

Sample	description	vo	conc. ʰg/ml	Total (mg)	recovery (%)
Ig06	Fraction III diluted + solvent/detergent	671	15,068	10,110	100
Ig07	Elution from SP resin	800	280	224	2.22
Ig09	Diafiltration	255	18.6	4.74	0.05
Ig11	Hydrophobic resin	275	<0.5	0.0	0.00

Sample	description	volume	conc. ʰg/ml	Total (mg)	recovery (%)
Ig06	Fraction III diluted + solvent/detergent	668	13842	9257	100
Ig07	Elution from SP resin	250	556	139	1.5
Ig09	Diafiltration	300	16.62	4.9	0.05
Ig11	Hydrophobic resin	275	<0.5	0.0	0.00

As can be seen from Table 6, the cationic exchange chromatography step is highly efficient in removing the detergent reagents that are added for the purpose of viral inactivation. More than 95% Triton levels were reduced by the cationic exchange chromatography. The following step of Diafiltration, brought the levels below the limits specified by the toxicological studies which are > than 50 PPM of Triton X-100 and 30 PPM of TnBP in the final product of 5 mg/ml. so that even without purification on

a hydrophobic column Triton levels were reduced to a medically acceptable level. Therefore it can be concluded that the hydrophobic chromatography on the C18 column is not essential but rather optional and was performed solely for the elimination of hydrophobic lipid or lipoproteins, and not for elimination of the solvent or detergent.

Example 5 Validation of solvent/detergent removal

A. Technical procedure

The source material for IVIg was Cohn Fraction III.

The fraction which contains about 3-5 mg/ml protein and 17% ethanol was concentrated by Diafiltration to 60-70 mg/ml. The salt and the ethanol were removed by the ration of 6 volumes of Diafiltration against water.

The pH was adjusted to 5.2 and solution is treated by solvent/detergent (0.3% Tri (n-butyl) phosphate, 1% Triton X-100 for 6 hours at 4°C).

The S/D reagents were removed by cationic exchange chromatography. At low pH (4.0) the positively charged IVIg is bound to the column.

The IVIg is removed from the cationic resin by increasing the salt concentration and increasing the pH to 7.0. Concentration of the protein to 70 mg/ml and removal of salt is done by Diafiltration against water.

In order to remove the lipids or the lipoprotein from the IVIg solution, the solution is passed through a hydrophobic column which is 10 times smaller than the cationic column.

B. Results

By utilizing the above procedure > 98% of the solvent/detergent were removed in the column spend.

The following is a flow chart describing the manufacturing process.

Flow Chart of the manufacturing process with In-process Controls

PRODUCTION STEP	SAMPLE NO.	PARAMETERS	RANGE
Fraction III	Ig01		
		Ethanol	16-18%
		Protein	3-5 mg/ml
→	**Ig02**		**LAL**
UF filtration against water			
→	**Ig03**		
(the concentration can be stored up to 4 weeks)		Protein	50-60 mg/ml
		TVC	$<10^4$ CFU/ml
(**Goal:** *removal of ethanol and salts*)		Ethanol	>1%
Adjustment of pH with 0.5 N NaOH		Osmolarity >	40 meq/l
pH admustment before SD addition			
→	**Ig04**		
		Protein	60-70 mg/ml
		pH	5.1-5.3
Addition of S/D reagents			
0.3% TnBP, 1% Triton X-100			
6 hours incubation at $6 \pm 2^{\circ}C$			
(virus inactivation by SD)			
→	**Ig05**		
		Triton	X-100 > 0.9%
		TnBP	>0.27%
Dilution of the SD containing solution to low protein containing			
→	**Ig06**		
		Protein	<50 mg/ml
		pH 5.1-5.3	
Wash the column with sodium			
Citrate pH = 4.0 (G2)			**LAL test**
→			
Loading on Cationic exchange SP-column (A)			
Elute the column with (G3)			
(*Goal: Elimination of S/D product and further purification*)			
→	**Ig07**		
		Protein	20-30 mg/ml
		Triton X-100	Qualitative
		TnBP	< 100 µg/ml
		pH	5.0-6.0
Ultralfiltration/diafiltration →	**Ig08**		**LAL test**
Concentration of the filtrate up to 50-70 mg/ml and			
difiltration against 6 volumes of water			
(*Goal: Elimination of sodium salts*			
and concentrating the elute)			
→	**Ig09**		

Protein	50-70 mg/ml
pH	4.5-5.5
Triton X-100	< 10 µg/ml
TnBP	< 10 µg/ml
Sodium	< 20 meq/L
Osmolarity	<30m Osmol/kg

→ **Ig10**

Loading on the SRD column (BO
Collection of elute (Goal: *Removal of lipo-proteins*)

→ **Ig11**

LAL

Protein	20-30 mg/ml
pH	4.0-5.0
pH	4.5-5.5
Triton X-100	< 10 µg/ml
density	0.95-1.051

Wash column SDR with ethanol (25-100%) and 2-Propanol
Addition of Maltose up to 10%
Adjustment pH at 3.95-4.05 by NaOH 0.5 M
Sterile filtration 0.22µ

→ **Ig12**

pH	3.9-4.05
Protein	50-70 g/l
density	> 1.03
osmolarity	>280mOsmol/Kg

Incubation 22 h at 37°C
(**Goal**: *Elimination of polymers*)

→ **Ig13**

TVC	$\leq 10^4$ CFU/ml

Ultrafiltration/Diafiltration → **Ig14**
Concentration of the filtrate up to 60-70 mg/ml and
difiltration against 3 volume of water
Addition of maltrost

→ **Ig15**

LAL test

pH	3.9-4.05
Osmolarity	200-300
Protein	>50 g/l
density	> 1.03

pH adjustment at 5.3-5.5 by NaOH 0.1 M
Addition of Maltrose 10%
(**Goal:** *Adjustment of pH*)

→ **Ig16**

Osmolarity	300 to 400
Sodium	< 20 meq/l
Protein	45-55 g/l\

	pH	5.3-5.5
	density	> 1.03
Filtration up to 0.2 μm		
Sterile filtration		
Purified bulk product		
Preparation of finished product		
Filling, Labeling, Storage at 2-6°C		
Quality Control Release		

This study was performed by removing the samples from the process steps of normal production batches, processed according to the Standard Manufacturing Procedure.

C. In process analysis

Quantitative determination of Triton X-100 was carried out by HPLC, while quantitative determination of TNBP was carried out by GC.

C1. Sampling method and sampling labeling

A sample from the S/D treated product was collected half an hour after the administration of the mixture. The product sampled was eluted in the final step of column A. A sample from the product which passed diafiltration was then collected and mixed well for 10 mins. before collection. A sample from the effluent of the column B was collected while noting the total weight of the vessel. Initially the effluent from the column loading was collected while continuing to collect until the absorbency reaches 5%. The volume of the solution collected was determined, the sample was mixed four times and 10 ml samples were obtained for TNPP and Triton. The samples were frozen at -18°C or colder until ready for assaying.

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Table 7

Sample No.	Sample Buffer	Sample Label
1	Sample Ig05 ½ after the addition of SD	Ig05
2	Sample Ig06 after dilution to < 50 mg/ml	Ig06
3	Sample Ig06a eluate	Ig06a
4	Ig07 Elution of fraction III from column A	Ig07
5	Ig09 after diafiltration and concentration	Ig09
6	Ig11 after SDR column	Ig11

C2 Results

The results are shown in the tables below

Batch Run 200b

Table 8

Removal of TNBP from IVIg

Sample No.	Label No.	Volume (L)	TNBP conc. (µg/ml)	TNBP total (mg)	TNBP (% of added)
1	Ig05	0.155	3319.44	514.5	100
2	Ig06	0.186	2466.28	458.7	89.15
3	Ig06a	0.161	1650.6	265.67	51.64
4	Ig07	0.511	126.73	64.39	12.5
5	Ig09	0.11	16.18	1.78	0.346
6	Ig11	0.1	< 5PPM	n.d.	n.d.

n.d. non-detectable (limit of detection = 2 µg/ml)

Table 9 - Removal of Triton X-100 from IVIg

Sample No.	Label No.	Volume (L)	Triton X-100 con. ((μg/ml)	Triton X-100 total (mg)	Triton X-100 (% of added)
1	Ig05	0.155	10620	1646.1	100
2	Ig06	0.186	7326.37	1362.7	82.78
3	Ig06a	0.161	7624.37	1227.52	74.57
4	Ig07	0.511	162.28	82.93	5.04
5	Ig09	0.11	37.37	4.11	0.25
6	Ig11	0.1	0.39	0.039	0.002

C3 Batch Run 200b

Table 10 - Removal of TNBP from IVIg

Sample No.	Label No.	Volume (L)	TNBP conc. (μg/ml)	TNBP total (mg)	TNBP (% of added)
1	Ig05	0.150	3161.74	474.26	100
2	Ig06	0.181	2447.39	442.98	93.33
3	Ig06a	0.163	1382.94	225.42	47.53
4	Ig07	0.446	126.62	56.47	11.90
5	Ig09	0.130	14.94	1.942	0.41
6	Ig11	0.121	< 5 PPM	n.d	

Table 11 - Removal of Triton X-100 from IVIg

Sample No.	Label No.	Volume (L)	Triton X-100 conc. (μg/ml)	Triton X-100 total (mg)	Triton X-100 (% of added)
1	Ig05	0.150	10104.25	1515.64	100
2	Ig06	0.181	7223.75	1307.50	86.28
3	Ig06a	0.163	7102.5	1157.71	76.38
4	Ig07	0.446	160.06	71.63	4.73
5	Ig09	0.130	38.15	4.96	0.33
6	Ig11	0.121	0.23	0.028	0.002

C4 Analysis of imput and output of solvent reagents of batch No. RUN 200b

Table 12

	Concentration (μg/ml)
Sample	
Initial TNBP	3319.44
Final TNBP	< 5 ppm
% Removal	> 99.85%
Initial Triton	10620
Final Triton	0.39
% Removal	> 99.99%

C5 Analysis of input and output of solvent reagents of batch No. RUN 200a

Table 13

	Concentration (μg/ml)
Sample	
Initial TNBP	3161.74
Final TNBP	< 5 ppm
% Removal	> 99.84%
Initial Triton	10104.25
Final Triton	0.23
% Removal	> 99.99%

D. Conclusion

From the above results, it can be seen that the cationic exchange chromatography step is highly efflciient in removing the solvent Detergent reagents that are added at the virus inactivation step. In the two trial batches, both the TNBP and Triton levels were reduced by greater than 99.8% to levels below the specified limits in the final product of 5 µg/ml and the tables of the SD elimination show consistency of the purification steps from batch to batch. Furthermore, it was proved that the hydrophobic column has only a marginal role in the solvent detergent removal and therefore it serves the sole purpose of elimination of lipids and lipoprotein from the product.

Example 6 Analysis of a batch of purified immunoglobulins

A batch of purified immunoglobulins prepared as above was transferred to Tel Ha Shomer Laboratory (Israel) for analysis.

Analysis of impurities indicated that the anti-complementary activity and prekallicrein activator were below limit levels indicating that the batches are suitable for clinical use.

Purified immunoglobulins prepared as described above were stored for 12 months at 4°C and at room temperature. Parameters tested remained stable during the year's storage at both temperatures.

Example 7 Clinical trial

8 children suffering from Hypogammaglobalinemia (lack of immunoglobulins) and 6 children suffering from multiple sclerosis were administered with a batch No. B-23161 prepared according to the method of the invention.

No abnormal results were evident hours after administration nor two weeks after administration.

CLAIMS:

1. A method for the purification of immunoglobulins from a source solution comprising:

- contacting the source solution with a cation exchange resin; and
- eluting the immunoglobulins bound to said cation exchange resin.

2. A method according to Claim 1 wherein the source colution is Cohn Fraction III.

3. A method according to Claim 1 or 2, wherein prior to contact with the cation exchange resin, the source solution is treated with an organic solvent and a detergent.

4. A method according to any one of Claims 1 to 3, wherein the cation exchange resin is present in a chromatographic column.

5. A method according to any one of Claims 1 to 3, wherein the cation exchange resins are pre-treated with an acidic solution.

6. A method according to Claim 5, wherein the acidic solution has a pH of 3.0-5.5.

7. A method according to Claim 6, wherein the acidic solution has a pH of 4.0-4.5.

8. A method according to any one of Claims 1 to 7, wherein the immunoglobulins are elutedby contacting the cation-exchange resin with a solution having a salt concentration to 0.3 M-1.5 M and a pH of 6.0-9.0.

9. A method according to any one of Claims 1 to 8, wherein the eluted immunoglobulins are passed through a hydrophobic column.

10. A method according to Claim 4 and 9, wherein the volume of hydrophobic column is 7 to 12 smaller than the cation-exchange column.

11. A method according to Claim 10, wherein the hydrophobic column is 10 times smaller than the cation exchange column.

12. A method according to Claim 1 for purification of immunoglobulins from Cohn Fraction III solution comprising:

(i) diafiltratring the Cohn Fraction III solution against water;

(ii) adjusting the pH of the diafiltrated solution to a pH of 4.0 to 5.5;

(iii) contacting the solution obtained in (ii) with an organic solvent and a detergent;

(iv) passing the solution obtained in (iii) through a cation exchange chromatographic column; and

(v) eluting the immunoglobulins absorbed on the column by passing a solution having a salt concentration of 0.3 M - 1.5 M and a pH of 6.0-7.0.

13. A method according to Claim 12, further comprising passing the eluted immunoglobulins on a hydrophobic column.

14. A method according to Claim 13, wherein the volume of the hydrophobic column is 7-12 times smaller than the cation-exchange column.

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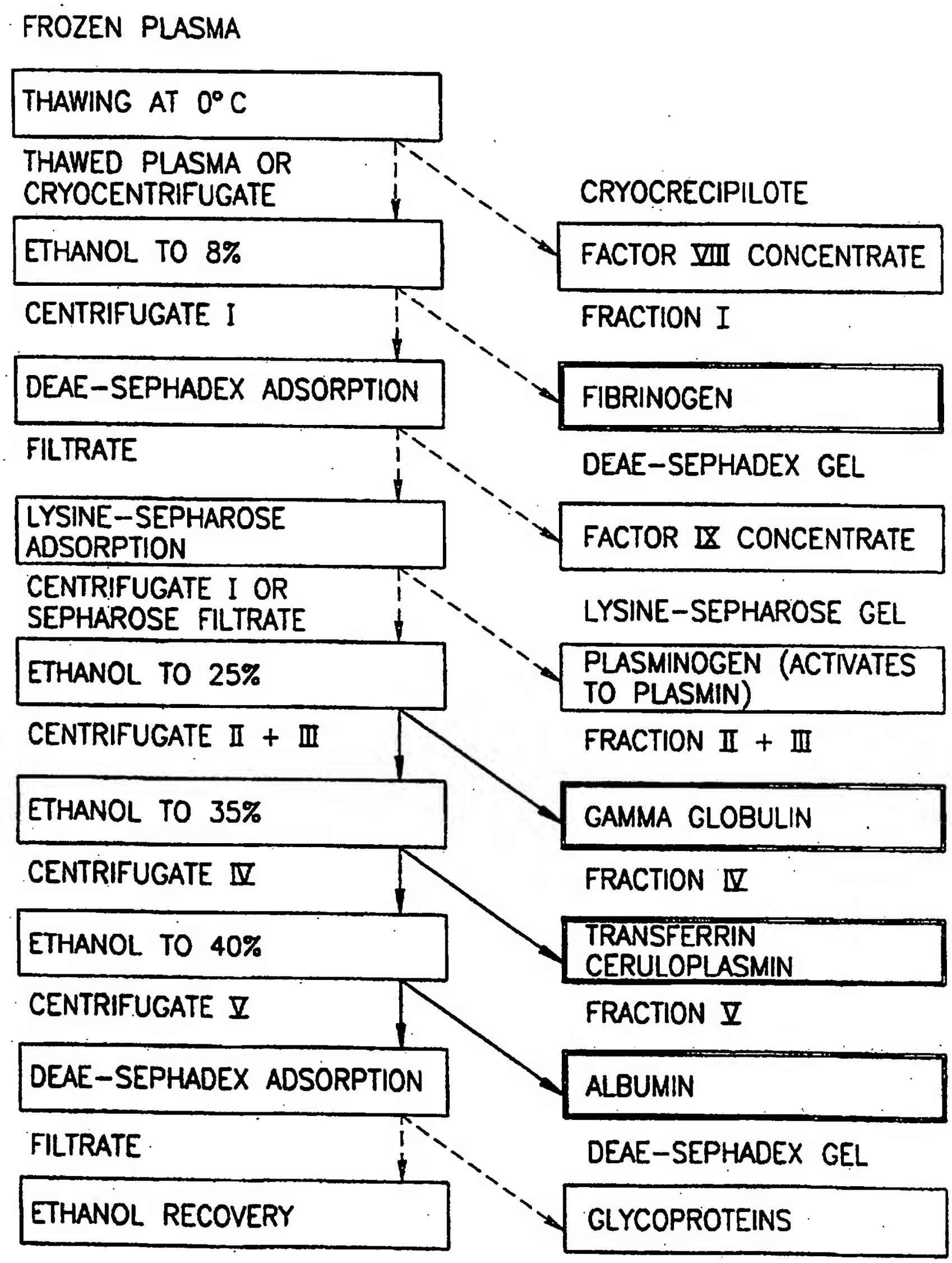

FIG.1

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/IL 98/00483

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/06 //C07K1/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 440 483 A (BAXTER INT) 7 August 1991 see column 1, line 41 - column 3, line 20 see column 4, line 6-11 - line 44-58 see column 5, line 35-51 see claim 1E ---	1-6,8,12
X	EP 0 530 447 A (BIOTEST PHARMA GMBH) 10 March 1993 see page 2, line 50 - page 3, line 10 see page 3, line 40 - page 4, line 15 see page 5; table II see page 6, line 14-18 ---	1,3-6, 8-11
X	US 4 877 866 A (RUDNICK DIETER ET AL) 31 October 1989 see example 8 see column 3, line 1-25 see column 4, line 61 - column 5, line 6 --- -/--	1,2,4,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 March 1999	26/03/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Covone, M

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INTERNATIONAL SEARCH REPORT

Intern. .al Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No
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A	EP 0 525 502 A (OCTAPHARMA AG) 3 February 1993 cited in the application see column 2, line 13-26	9-11,13, 14

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Information on patent family members

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